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The ameliorative effects of 24-epibrassinolide on shoot organogenesis inhibition occurring under NaCl-stressed conditions in cultures of cotyledon and hypocotyl explants of tomato (*Lycopersicon esculentum* Mill.)

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Abstract – In this study, possible effects of 24-epibrassinolide (24-epiBL) pretreatment against NaCl stress were investigated using *in vitro* shoot organogenesis from cotyledon and hypocotyl explants in M-28 tomato hybrid cultivar. The cotyledon and hypocotyl explants of 10-day sterile seedlings were treated with 1 μM and 2 μM 24-epiBL solutions (prepared with 70% acetone) for 30 seconds and applied explants were cultured on MS medium supplemented with 2 mg/l 6-benzylaminopurine (BAP) + NaCl (0, 20, 40, 60, 80 and 100 mM). It was determined that the regeneration percentage as well as the shoot number/explant, the shoot length and the leaf number/shoot derived from both explants suffered NaCl stress after 30 days. 24-epiBL pretreatment against NaCl stress showed ameliorative effects and hypocotyl explants gave better results than cotyledon explants for these growth parameters. Different stages of shoot organogenesis from hypocotyl explants applied with 24-epiBL under salt stress were observed with scanning electron microscopy. As a result, it was shown that 24-epiBL treatment against NaCl stress may play an effective role in salt tolerance in M-28 tomato hybrid cultivar.

Keywords: 24-epibrassinolide, *in vitro* culture, NaCl stress, shoot organogenesis, tomato.

Introduction

Salinity in soil and irrigation water is one of the environmental problems that have the most negative impact on agricultural productivity in arid and semi-arid areas (Mohamed et al. 2010). High salinity influences plant growth and development due to degradation of or damage to cellular macromolecules such as lipids, proteins and nucleic acid (Aly et al. 2012).

In vitro plant tissue culture techniques are very useful and economical tools for the study of the physiological effects of NaCl at cellular level under controlled conditions, and they give us valuable information concerning the responses of plants to NaCl stress (Cano et al. 1998, Mercado et al. 2000). Additionally, tissue culture can dissolve various limitations triggered by salt stress (Osman et al. 2010). In vitro techniques may be used quickly to screen a lot of genotypes in plant culture under stress conditions (Aazami et al. 2010). Various tissue techniques can be utilized to derive NaCl tolerant cell lines and to improve tolerance to salt stress in several plants such as tomato (El-Enany 1995).

The organogenesis process is shoot or plantlet formation from cell, tissue or callus, and there is a positive interaction between plantlet formation and callus development in tomato (Noaman and Ahmad 2004). The success of the regeneration response in tomato was reported to depend on genotype, explants, and plant growth regulators in culture media (Bhatia et al. 2004, Yilmaz and Burun 2014). There have been some studies about negative effects on direct and indirect regeneration of tomato explants affected by the stress of rising NaCl concentration (Mercado et al. 2000, Hassanein 2004, Mohamed et al. 2011).

Brassinosteroids, which exist naturally in plants, are a new class of steroidal plant hormones (Fujioka et al. 1998). They affect numerous physiological processes such as cell elongation and proliferation when applied exogenously at nanomolar or micromolar concentrations (Clouse 1996). In addition to their roles in plant development, brassinosteroids have preventative effects against salt stress and various abiotic stress factors and have given satisfactory results in reducing the effects of environmental stress in many horticulture crops (Surgun et al. 2012).

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In vitro shoot regeneration from explants such as hypocotyl, cotyledon, leaf and shoot tip has already been achieved in tomato and other economically important plants in saline conditions (Mercado et al. 2000, Hassanein 2004, Noaman and Ahmad 2004, Mohamed et al. 2011). However, there is no literature concerning the study of the possible effects of brassinosteroids on shoot regeneration under salt stress. The purpose of this study is to investigate for the first time the effect of short-term exogenous 24-epiBL pretreatment against salt stress using in vitro shoot organogenesis in tomato.

Material and methods

Plant material and *in vitro* culture

Tomato M-28 hybrid cultivar was used as plant material in this study and its seeds were obtained from Agrotek Seed Agriculture Industry and Trade Limited Company. For surface sterilization, the seeds were soaked in 2.25% NaOCl (50% diluted Na-hypochlorite) for 5 minutes and then thoroughly washed with sterile distilled water three times (Yilmaz-Gokdogan and Burun 2015). These seeds were germinated on half-strength Murashige-Skoog (1962) (½ MS) medium supplemented with 20 g L^{-1} sucrose and 7 g L^{-1} agar. The cotyledon (5 mm × 5 mm) and hypocotyl (5 mm) explants of 10-day sterile seedlings were treated with 1 µM and 2 µM 24-epibrassinolide (24-epiBL) solutions prepared with 70% acetone for 30 seconds and the control explants were exposed to only 70% acetone application. 24-epiBL applied explants were transferred to MS medium including 2 mg L^{-1} 6-benzylaminopurine (BAP) and increasing concentrations (0, 20, 40, 60, 80 and 100 mM) of NaCl (Yilmaz and Burun 2014). pH of MS medium containing 30 g L^{-1} sucrose was adjusted to 5.8 and then 7 g L^{-1} agar was added. The sterilization of MS medium was carried out in an autoclave at 121 °C and under 1 atm pressure for 15 minutes. All the culture vessels were kept under a 16 hour photoperiod (45 µE m^{-2} s^{-1} supplied by daylight fluorescent tubes) at 25 °C ± 2 in a culture room. For each application, 20 cotyledon and 20 hypocotyl explants were used and each treatment was repeated at least two times in the study. The regeneration percentage (%), the shoot number/explants, the shoot length (mm) and the leaf number/shoot derived from both explants were evaluated after four weeks.

Scanning electron microscopy examination (SEM)

The different stages of direct and indirect shoot organogenesis from hypocotyl explants in the various samples were investigated by SEM at the end of in vitro culture. 0.5 to 1 cm samples were taken at areas where shoot organogenesis may be formed, and fixation was conducted in 2.5% gluteraldehyde and 0.1 M phosphate buffered saline solution (pH 7.4), OsO_4 (osmium tetraoxide). After dehydration at first through a graded ethanol series and then ethanol: amyl acetate series, samples were finally soaked in pure amyl acetate, dried via CO_2 critical point drying. SEM analyses were carried out at the Research and Application Center for the Research Laboratory of Mugla Sitki Kocman University.

Data collection and statistical analysis

All experiments were repeated at least two times and standard errors of the means were calculated. Data were analyzed by using Statistica statistical program package. All data obtained were subjected to analysis of variance (one-way ANOVA). Factor analysis of variance was performed to examine the effect of the explant type, 24-epiBL pretreatment and their interaction on the investigated parameters. Comparisons with P-values ≤ 0.05 were considered significantly different.

Results

The study was performed according the effect of 24-epiBL of NaCl stress evaluated on in vitro shoot regeneration and plantlet formation. It was found that the regeneration percentage in 24-epiBL non-treated cotyledon explants was the highest in the NaCl-free control (Fig. 1). Increasing NaCl concentrations negatively affected regeneration percentage, ranging from 90% to 3% (from 0 to 80 mM NaCl, respectively). The appearance of buds and development of leaves from 24-epiBL treated and non-treated cotyledon explants were observed after two weeks in culture media. In addition both direct and indirect shoot organogenesis occurred from cotyledons explants (Fig. 2). When we evaluated the effect of 24-epiBL treatment against NaCl stress, both 24-epiBL treatments at 20 mM NaCl, 1 µM 24-epiBL at 40 mM NaCl and 80 mM NaCl showed statistically positive effects on regeneration percentage (Figs. 1 and 2); on the other hand, 24-epiBL treated cotyledon explants did not regenerate under a 100 mM NaCl condition (data not shown).

Similarly, regeneration percentage decreased in 24-epiBL non-treated the hypocotyl explants under salt stress (Fig. 3). In these explants, the generation percentage was 96% in the NaCl-free control medium and it was also determined that regeneration was76%, 54%, 34%, 7%, 10% from 20 mM NaCl to 100 mM NaCl. In general there was a gradual decrease for the regeneration percentage from 0 to 100 mM NaCl, but a particularly clear decrease in the regeneration percentage was observed at 80 mM and 100 mM NaCl. In the all NaCl concentrations, regeneration in hypocotyl explants was higher than in cotyledon explants. Adventive shoots with leaves were observed from control to 60 mM NaCl, but leaf formation did not occur at 80 mM and 100 mM NaCl. Direct and indirect shoot organogenesis occurred from cotyledon explants for all treatments (Fig. 4). When the effect of 24-epiBL against NaCl stress was evaluated in this study, the increase of the regeneration percentage with 1 µM 24-epiBL pretreatment at 60 mM and 80 mM NaCl was statistically significant (Fig. 3). The factorial analysis showed that the explant type at 60, 80 and 100 mM NaCl concentrations had a significant effect on regeneration. 24-EpiBL pretreatment, on the other hand, except with 100 mM NaCl, affected regeneration at all the NaCl concentrations (On-line Suppl. Tab. 1). Explant type × 24-epiBL interaction was found to lead to statistically significant differences at 20, 40, 60 and 100 mM NaCl concentrations.

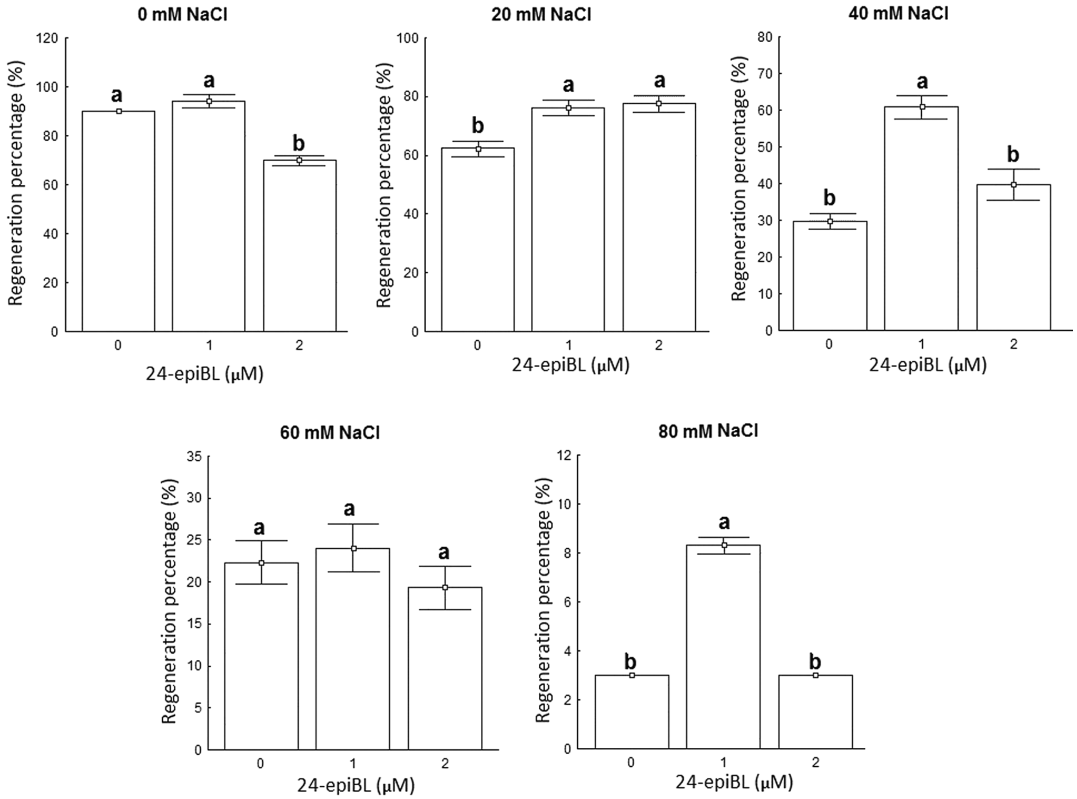

Fig. 1. The regeneration percentage (%) in cotyledon explants of *Lycopersicon esculentum* pretreated with 24-epibrassinolide (24-epiBL) in concentrations 0, 1 and 2 μM and NaCl in the range of concentrations 0–80 mM. Different letters show the statistically significant differences between 24-epiBL pretreatments, at $p \leq 0.05$.

Fig. 2. The regeneration status of 24-epibrassinolide treated and non-treated cotyledon explants of *Lycopersicon esculentum* in MS medium containing 20, 40 and 80 mM NaCl after 30 days: a) 20 mM NaCl + 0 μM 24-epiBL, b) 20 mM NaCl + 1 μM 24-epiBL, c) 20 mM NaCl + 2 μM 24-epiBL, d) 40 mM NaCl + 0 μM 24-epiBL, e) 40 mM NaCl + 1 μM 24-epiBL, f) 40 mM NaCl + 2 μM 24-epiBL, g) 80 mM NaCl + 0 μM 24-epiBL, h) 80 mM NaCl + 1 μM 24-epiBL, i) 80 mM NaCl + 2 μM 24-epiBL. Bars = 1 cm.

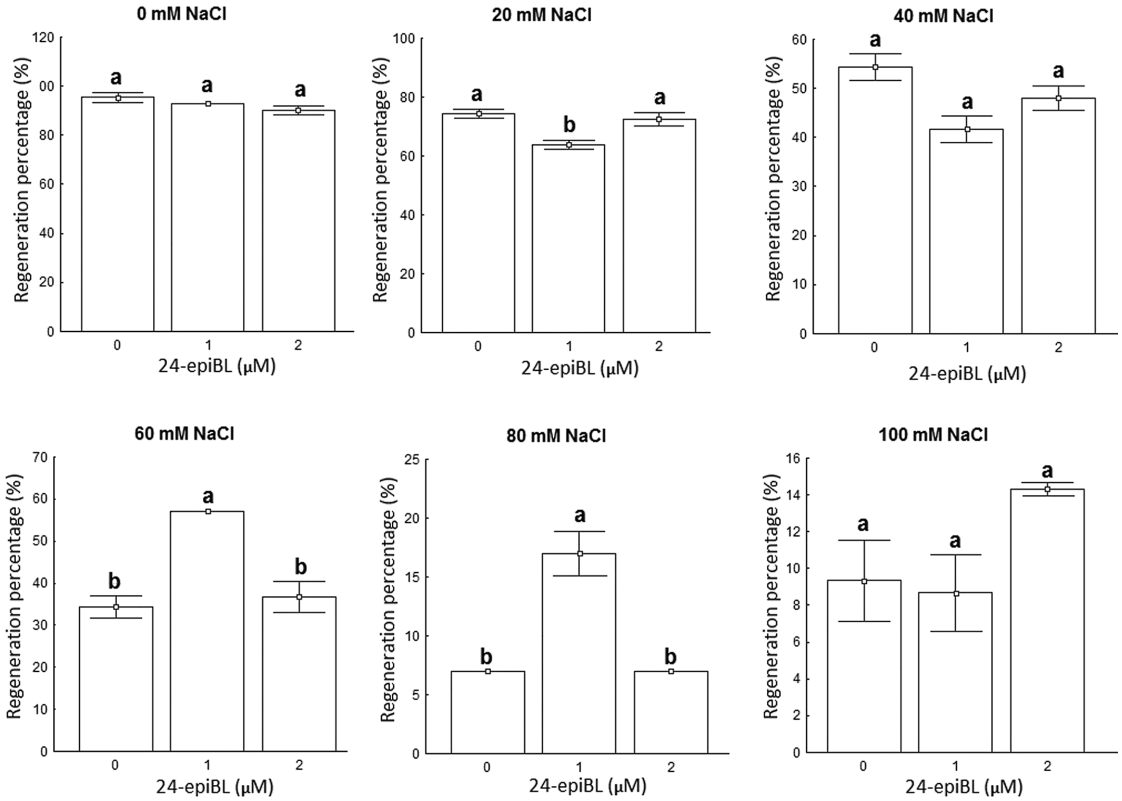

Fig. 3. The regeneration percentage (%) in hypocotyl explants of *Lycopersicon esculentum* pretreated with 24-epibrassinolide (24-epiBL) in concentrations 0, 1 and 2 µM and NaCl in the range of concentrations 0–100 mM. Different letters show the statistically significant differences between 24-epiBL pretreatments, at $p \leq 0.05$.

Fig. 4. The regeneration status of 24-epibrassinolide treated and non-treated hypocotyl explants of *Lycopersicon esculentum* in MS medium containing 60, 80 and 100 mM NaCl after 30 days: a) 60 mM NaCl + 0 µM 24-epiBL, b) 60 mM NaCl + 1 µM 24-epiBL, c) 60 mM NaCl + 2 µM 24-epiBL, d) 80 mM NaCl + 0 µM 24-epiBL, e) 80 mM NaCl + 1 µM 24-epiBL, f) 80 mM NaCl + 2 µM 24-epiBL, g) 100 mM NaCl + 0 µM 24-epiBL, h) 100 mM NaCl + 1 µM 24-epiBL, i) 100 mM NaCl + 2 µM 24-epiBL. Bars = 1 cm.

Besides regeneration percentage, adventive shoot number of per explant (Tab. 1), shoot length (Tab. 2) and leaf number of per shoot (Tab. 3) were also evaluated. In the 24-epiBL non-treated cotyledon explant, shoot number of per explant was very close from 0 to 40 mM NaCl, but it decreased from 60 mM to 100 mM NaCl. The highest shoot number was interestingly observed with the 2 μM 24-epiBL pretreatment at 80 mM NaCl (11.00/explant). The shoot number decreased in the 24-epiBL non-treated at 80 and 100 mM NaCl (1.00/explant) and there was no shoot formation with 24-epiBL treatment at 100 mM NaCl. In this study, only the effect of 2 μM 24-epiBL pretreatment against 80 mM NaCl stress was found statistically different (Tab. 1). Shoot number derived from 24-epiBL non-treated and treated hypocotyl explants on MS medium was negatively affected when salt stress was increased. When we evaluated the effect of 24-epiBL against NaCl stress, only 1 μM 24-epiBL treatment at 60 mM NaCl was statistically significant (Tab. 1). The explant type used was found to have a significant effect on the adventive shoot number/explant at 0, 20 and 100 mM NaCl concentrations (data not shown). 24-EpiBL pretreatement led to significant differences at 80 mM NaCl concentration while explant type × 24-epiBL interaction did not have a significant effect.

The shoot length derived from 24-epiBL non-treated cotyledon explants was observed to increase from 0 mM NaCl to 80 mM NaCl. The shoot length was not measured because the regenerated shoot swere very small (< 0.5 mm) at 100 mM NaCl. Additionally, no effect of 24-epiBL pretreatment against NaCl stress was determined on the shoot length (Tab. 2). In 24-epiBL treated and non-treated hypocotyl explants, the adventive shoot length was high for all the NaCl concentrations when the compared with the control. When it was evaluated the effect of 24-epiBL under salt stress, the positive effect of 1 μM 24-epiBL treatment was found statistically significant only at 100 mM NaCl (Tab. 2). Also, the effects of explant type, 24-epiBL and explant type × 24-epiBL interaction on the shoot length were statistically evaluated; it was found that explant type has a significant effect on the shoot length at 0, 60 and 100 mM NaCl concentrations, 24-epiBL led to significant differenc-

Tab. 1. The number of 30-day adventive shoots of *Lycopersicon esculentum* derived from cotyledon and hypocotyl explants (shoot number/explant) upon 24-epibrassinolide (24-epiBL) application and NaCl in the range concentrations 0–100 mM. Values are means ± standard errors. Different letters show the statistical difference between 24-epiBL treatments and the control in the same column, at P≤0.05.

24-EpiBL (μM)	Shoot number/cotyledon explant					
	NaCl (mM)					
	0	20	40	60	80	100
0	8.14±1.32[a]	9.17±1.79[a]	8.27±2.28[a]	5.87±2.25[a]	1.00±0.00[b]	1.00±0.00[a]
1	7.80±1.18[a]	9.08±1.45[a]	5.54±1.29[a]	5.37±1.74[a]	2.66±0.33[b]	0.00±0.00[a]
2	8.00±1.63[a]	8.82±1.49[a]	6.93±2.24[a]	2.87±0.49[a]	11.00±0.57[a]	0.00±0.00[a]
24-EpiBL (μM)	Shoot number/hypocotyl explant					
	NaCl (mM)					
	0	20	40	60	80	100
0	20.30±2.37[a]	13.50±1.68[a]	6.12±0.95[a]	3.92±0.63[b]	4.33±1.45[a]	2.50±1.19[a]
1	24.82±2.64[a]	12.79±1.82[a]	7.29±1.52[a]	8.41±1.35[a]	3.87±1.10[a]	3.25±1.60[a]
2	22.22±2.56[a]	12.75±1.52[a]	7.81±1.38[a]	5.88±0.79[ab]	9.66±4.97[a]	4.00±1.15[a]

Tab. 2. The length of shoots derived from cotyledon and hypocotyl explants of *Lycopersicon esculentum* upon 24-epibrassinolide (24-epiBL) pretreatment and NaCl treatment in the range of concentrations 0–100 mM. Values are means ± standard errors. Different letters show the statistical difference between 24-epiBL treatments and the control in the same column, at P≤0.05.

24-EpiBL (μM)	Length of shoots derived from cotyledon					
	NaCl (mM)					
	0	20	40	60	80	100
0	4.73±0.32[a]	7.45±0.54[a]	9.89±1.05[a]	7.53±1.67[a]	6.00±0.00[a]	0.00±0.00[a]
1	4.13±0.34[a]	6.45±0.76[a]	6.00±0.73[b]	5.57±1.04[a]	1.00±0.00[b]	0.00±0.00[a]
2	4.81±0.44[a]	6.49±0.51[a]	7.27±0.94[ab]	1.00±0.00[a]	4.66±0.66[a]	0.00±0.00[a]
24-EpiBL (μM)	Length of shoots derived from hypocotyl					
	NaCl (mM)					
	0	20	40	60	80	100
0	3.29±0.09[a]	6.84±0.31[a]	9.07±0.72[a]	8.22±0.97[a]	10.00±2.51[a]	3.50±0.95[b]
1	3.18±0.09[a]	7.77±0.47[a]	7.10±0.79[a]	8.22±0.70[a]	8.92±1.86[a]	8.33±2.33[a]
2	3.37±0.11[a]	7.17±0.40[a]	9.47±0.73[a]	5.69±0.59[b]	5.00±0.57[a]	3.90±0.73[b]

Tab. 3. The number of leaf per shoot derived from cotyledon and hypocotyl explants of *Lycopersicon esculentum* upon 24-epibrassinolide (24-epiBL) pretreatment and NaCl treatment in the range of concentrations 0–100 mM. Values are means ± standard errors. Different letters show the statistical difference between 24-epiBL treatments and the control in the same column, at P≤0.05**.**

24-EpiBL (μM)	The number of leaf per shoot derived from cotyledon					
	NaCl (mM)					
	0	20	40	60	80	100
0	1.14±0.02[a]	1.18±0.03[a]	1.17±0.05[a]	1.23±0.12[a]	1.00±0.00[a]	0.00±0.00[a]
1	1.08±0.02[a]	1.12±0.03[a]	1.22±0.08[a]	1.36±0.13[a]	1.00±0.00[a]	0.00±0.00[a]
2	1.16±0.04[a]	1.16±0.03[a]	1.24±0.08[a]	1.00±0.00[a]	1.33±0.33[a]	0.00±0.00[a]
24-EpiBL (μM)	The number of leaf per shoot derived from hypocotyl					
	NaCl (mM)					
	0	20	40	60	80	100
0	1.01±0.004[b]	1.10±0.01[b]	1.26±0.05[a]	1.20±0.07[a]	1.33±0.33[a]	1.00±0.00[a]
1	1.01±0.004[b]	1.19±0.04[ab]	1.25±0.08[a]	1.37±0.08[a]	1.07±0.07[a]	1.00±0.00[a]
2	1.08±0.013[a]	1.25±0.04[a]	1.17±0.05[a]	1.38±0.09[a]	1.33±0.21[a]	1.11±0.11[a]

es at 40 and 60 mM NaCl concentrations, but explant type × 24-epiBL interaction was found to be not significantly different (On-line Suppl. Tab. 2).

The leaf number per shoot derived from 24-epiBL treated and non-treated cotyledon explants was very close and there was no statistically significant effect of 24-epiBL against NaCl stress (Tab. 3). The leaf number of per shoot developed from 24-epiBL non-treated hypocotyl explants, like cotyledon explants, ranged from 1.00 to 1.33. The positive effect of 2 μM 24- epiBL treatment under 0 mM and 20 mM NaCl stress was statistically significant on the leaf number per shoot (Tab. 3). When the effects of explant type, 24-epiBL and explants type × 24-epiBL interaction on the leaf number of per shoot according to NaCl concentrations were statistically evaluated the explant type was found to have a significant effect at 0 mM and 100 mM NaCl. 24-EpiBL pretreatment, on the other hand, led to significant differences only in the control. Explant type × 24-epiBL interaction was found to have a significant effect at 20 mM NaCl (On-line Suppl. Tab. 3).

Different stages of regeneration were observed from 24-epiBL treated and non-treated hypocotyl explants by SEM at the end of 30-day in vitro culture. SEM showed that direct regeneration process started by formation of organized cell groups (Fig. 5a) and the regenerated shoots with first leaf primordia formed from these cell groups (Figs. 5b and 5c). Finally the development of regenerated shoots and adventive shoots with fully formed leaf primor-

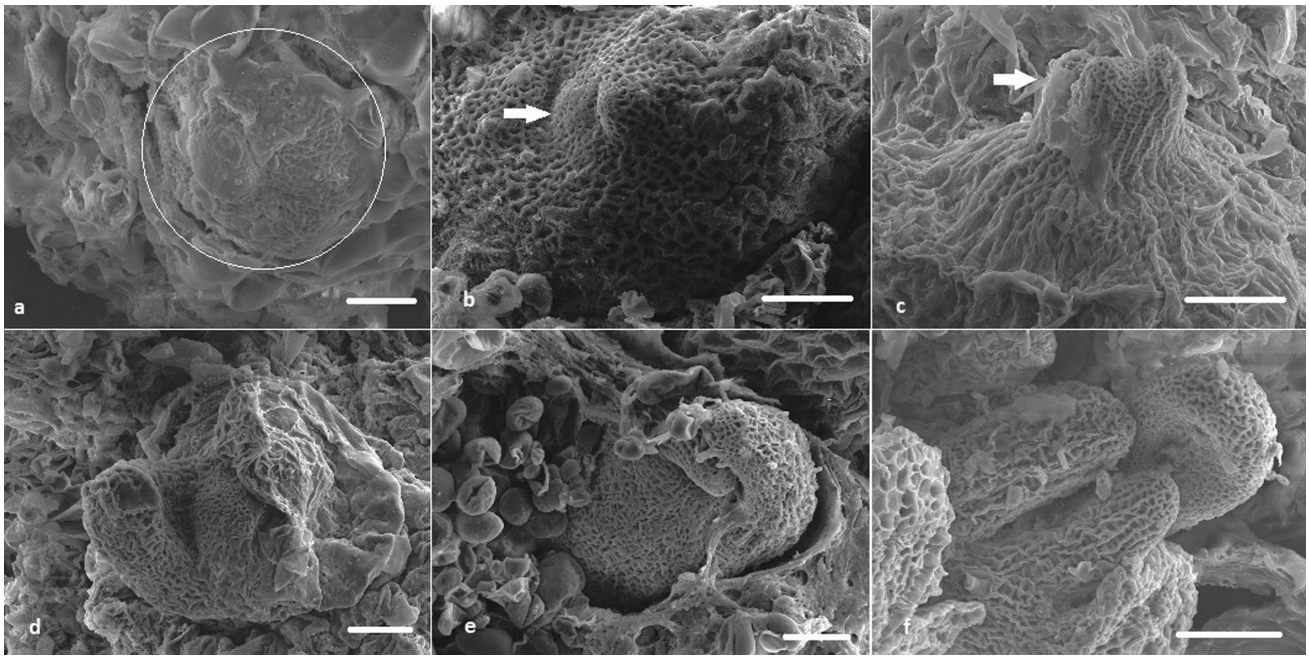

Fig. 5. Different stages of adventive shoot initiation from hypocotyl explants of *Lycopersicon esculentum* with or without the pretreatment with 24-epibrassinolide under salt stress after 30 days in culture: a) 100 mM NaCl + 1 μM 24-epiBL, b) 60 mM NaCl + 1 μM 24-epiBL, c) 20 mM NaCl + 0 μM 24-epiBL, d) 60 mM NaCl + 0 μM 24-epiBL, e) 80 mM NaCl + 1 μM 24-epiBL, f) 0 mM NaCl + 0 μM 24-epiBL; circle denotes organogenic callus, and arrow denotes leaf primordia at different developmental stages. Bars = 100 μm.

dia was observed (Figs. 5d–f). In addition to SEM, mophological observations too revealed that NaCl stress did not affect the shoot organogenesis process from hypocotyl explants. But NaCl stress depressed shoot initiation and development, and NaCl stress (especially 80 mM and 100 mM NaCl) inhibited leaf formation in adventive shoots (Figs. 4 and 5a–f).

Discussion

In this study, at first the effects of 24-epiBL pretreatment on shoot growth were evaluated using in vitro shoot regeneration under NaCl stress. In vitro shoot morphogenesis on tomatoes is the most important method used for the evaluation and screening of NaCl tolerance. Increased salinity leads to reduction of shoot number, shoot length, fresh weight and dry weight (Mercado et al. 2000). Suppressed shoot growth and development because of high salinity are connected with water stress, specific ion toxicity and ion imbalance or induced nutritional deficiency. Furthermore, the highest concentration of Na^+ might cause problems to membrane stability, enzyme inhibition, and cell division and elongation defects (Aly et al. 2012). Especially, reduction in growth parameters such as callus fresh weight, organogenic shoot induction and leaf formation might be the result of reduced water availability in the culture media with NaCl (Chamandoosti 2007). In this study, it was determined that the shoot regeneration percentage in the cotyledon and hypocotyl explants was high in salt free medium but shoot regeneration capacity of tested explants was inhibited by increasing NaCl stress. 24-EpiBL pretreatment used for obtaining salt tolerance was conducted on cotyledon and hypocotyl explants, and 24-epiBL against various NaCl concentrations showed ameliorative effects. Similarly Mercado et al. (2000) determined that regeneration from leaf disc explants was high under salt free condition in tomato Pera and HF cultivars and suffered under increasing salt stress. Benderradji et al. (2012) reported that callus induction, shoot proliferation from callus and shoot regeneration under salt stress negatively affected in vitro embryo culture in wheat. These findings show that shoot regeneration could depend on tomato cultivar, genotype and explant type under increasing NaCl stress.

The number of regenerated shoots derived from 24-epiBL treated and non-treated cotyledon and hypocotyl explants was high under salt free medium and it decreased under salt stress. The shoot number increased with 24-epiBL treatment under salt stress. Mohamed et al. (2011) reported that the shoot number decreased in cotyledon and hypocotyl explants of tomato Pearl and Beril cultivars in saline conditions. In addition, Chamandoosti (2007) showed that adventive shoot number from canola hypocotyl segments decreased under stress conditions. These findings show that NaCl stress adversely affects shoot organogenesis. The shoot length from both explants increased also from 0 to 100 mM NaCl concentration in this study. However, Mohamed et al. (2011) reported that the shoot length decreased under salt stress in Pearl and Beril tomato cultivars.

The results showed that NaCl stress greatly influenced the in vitro performance of two different explants of tomato M-28 cultivars and that shoot induction and development was negatively affected by increasing NaCl stress. The physiological processes such as decline in photosynthesis, change in cell turgor, metabolite accumulation, increase in reactive oxygen species and changes in antioxidative enzyme activity to scavenging of these reactive oxygen species, disturbance of carbon and nitrogen allocation and change in ion homeostasis occurring under NaCl stress are well known; recently it has been very popular to use various biologically active substances (proline, polyamine, plant growth regulators especially brassinosteroids) to understand the relationships among these physiological events for obtaining salt tolerance.

In this study, the positive effects of 24-epiBL treatment were firstly determined on in vitro shoot regeneration from 24-epiBL treated cotyledon and hypocotyl explants under salt stress. Additionally hypocotyl explants had better results than cotyledon explants on all parameters tested such as regeneration percentage, shoot number and shoot length. SEM analysis showed that no differences relating to the morphology of the shoot organogenesis from hypocotyl explants were observed by NaCl and 24-epiBL treatments. 24-epiBL pretreatments can improve shoot growth and development under NaCl stress and may play an effective role for salt tolerance in M-28 tomato hybrid cultivar.

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